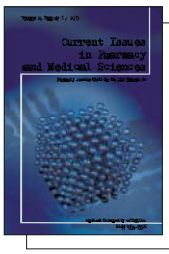

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Influence of N-(p-acetylphenyl)-p-isopropoxyphenylsuccinimide on the protective action of classical antiepileptic drugs against maximal electroshock-induced seizures in mice

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ABSTRACT

The aim of this study was to determine the effects of N-(p-acetylphenyl)-p-isopropoxyphenylsuccinimide (APIPPS) on the protective action of four classical antiepileptic drugs (AEDs: carbamazepine [CBZ], phenobarbital [PB], phenytoin [PHT] and valproate [VPA]) in the maximal electroshock (MES)-induced seizures in mice. Tonic hind limb extension (seizure activity) was evoked in adult male albino Swiss mice by a current (25mA, 500V, 50Hz, 0.2s stimulus duration) delivered *via* auricular electrodes. Total brain AED concentrations were measured with fluorescence polarization immunoassay to ascertain whether any observed effects were consequent to a pharmacodynamic and/or a pharmacokinetic interaction between APIPPS and classical AEDs. Results indicate that APIPPS administered *intraperitoneally* at a dose of 150 mg/kg significantly elevated the threshold for electroconvulsions in mice. APIPPS at lower doses of 25, 50 and 100 mg/kg had no impact on the threshold for electroconvulsions in mice. Moreover, APIPPS at 100 mg/kg significantly enhanced the anticonvulsant activity of PB and VPA, but not that of CBZ or PHT, in the MES test in mice. APIPPS at a dose of 50 mg/kg significantly potentiated the anticonvulsant action of VPA, but not that of PB in the mouse MES model. Pharmacokinetic experiment revealed that APIPPS did not alter total brain concentrations of PB or VPA in mice. Summing up, the enhanced anticonvulsant action of PB and VPA by APIPPS in the mouse MES model and lack of pharmacokinetic interactions between drugs, make the combinations of APIPPS with PB and VPA of importance for further experimental and clinical studies. The combinations of APIPPS with CBZ and PHT are neutral from a preclinical viewpoint.

Keywords: antiepileptic drugs, maximal electroshock-induced seizures, pharmacokinetic / pharmacodynamic interaction, p-isopropoxyphenylsuccinimide derivative

INTRODUCTION

In experimental epileptology, to detect a substance possessing anticonvulsant activity, a huge number of compounds undergo examination in the first anticonvulsant screening test in rodents [12, 13]. The first rapid screening of potential anticonvulsant compounds is routinely performed in the mouse maximal electroshock (MES)-induced seizure model [12,13].

Experimental evidence indicates that some succinimide derivatives possess clear anticonvulsant properties in *in vivo* screening tests in rodents [2,3,8-10,14]. For instance, N-morpholinemethyl derivative of m-bromophenylsuccinimide [3], N-pyridyl-substituted succinimides [14], 3-cyclohexylsuccinimides [2], N-(anilinomethyl)-p--isopropoxyphenylsuccinimide (AMIPPS) [10], p-isopropoxyphenylsuccinimide monohydrate (IPPS) [9], N-(ortho-carboxyanilinomethyl)-p-isopropoxyphenylsuccinimide (o-CAMIPPS), N-(meta-carboxyanilinomethyl)-p-isopropoxyphenylsuccinimide (m-CAMIPPS), and N-(para-carboxyanilinomethyl)-p-isopropoxyphenylsuccinimide (p-CAMIPPS) [8], exhibited potent anticonvulsant effects in the MES test in mice, recognized as the most widely employed animal seizure model for early identification of candidate anticonvulsant drugs.

In our pilot study, we found that N-(p-acetylphenyl)-p--isopropoxyphenylsuccinimide (APIPPS) possesses anticonvulsant properties by suppressing tonic-clonic sei-

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zures in mice. We sought, therefore, to evaluate the effect of APIPPS on the threshold for electroconvulsions and to assess its influence on the protective activity of four classical antiepileptic drugs (AEDs: carbamazepine [CBZ], phenobarbital [PB], phenytoin [PHT] and valproate [VPA]) in the mouse MES model. The threshold for electroconvulsions and the MES test are both thought to be experimental models of tonic-clonic seizures and, to a certain extent, of partial convulsions with or without secondary generalization in humans [5,6]. In these experimental tests one can readily evaluate the antiseizure potential of agents and compounds possessing anticonvulsant properties and determine their effects on classical and second-generation AEDs, which are fully effective in the suppression of tonic-clonic seizures in humans [5,6]. Therefore, it was appropriate to use both tests to evaluate the effects of APIPPS. Finally, total brain AED concentrations were measured with fluorescence polarization immunoassay to ascertain whether any observed effects were consequent to a pharmacodynamic and/or a pharmacokinetic interaction.

MATERIALS AND METHODS

Animals and experimental conditions. Adult male Swiss mice (weighing 22–26 g) that were kept in colony cages with free access to food and tap water, housed under standardized housing conditions (natural light-dark cycle, temperature of 23 ± 1°C, relative humidity of 55 ± 5%), were used. After seven days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups each comprising eight mice. Each mouse was used only once and all tests were performed between 08:00 a.m. and 03:00 p.m. Procedures involving animals and their care were conducted in accordance with current European Community and Polish legislation on animal experimentation. Additionally, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. All experiments were carried out according to the National Institute of Health Guidelines for the care and use of laboratory animals and the European Council Directive on 24 November 1986 for Care and Use of Laboratory Animals (86/609/EEC), and approved by the First Local Ethics Committee at the Medical University of Lublin (License no.: 18/2006) and the Second Local Ethics Committee at the University of Life Sciences in Lublin (License no.: 79/2009).

Drugs. The following drugs were used: APIPPS (N-(p-acetylphenyl)-p-isopropoxyphenylsuccinimide [M.W.: 351.387] – synthesized by Dr. S.L. Kocharov, Mndjoyan's Institute of Fine Organic Chemistry of the National Academy of Sciences of the Republic of Armenia, Yerevan, Armenia), carbamazepine (CBZ – a gift from Polpharma, Starogard Gdański, Poland), phenobarbital (PB – Polfa, Kraków, Poland), phenytoin (PHT – Polfa, Warszawa, Poland) and valproate (VPA – magnesium salt - kindly donated by ICN-Polfa S.A., Rzeszów, Poland). All drugs, except for VPA, were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in distilled water, while VPA was directly dissolved in distilled water. All drugs were administered intraperitoneally (ip) as a single injection, in a volume of 5 ml/kg body weight. Fresh drug solutions were prepared on each day of experimentation and administered as follows: PHT - 120 min, PB and APIPPS - 60 min, CBZ and VPA – 30 min before electroconvulsions, motor coordination, grip-strength and long-term memory tests and before brain sampling for the measurement of AED concentrations. The pretreatment times before testing of the AEDs were based upon information about their biological activity from the literature and our previous experiments [8-10]. The times to the peak of maximum anticonvulsant effects for all AEDs were used as the reference times in all behavioral tests and pharmacokinetic estimation of brain AED concentrations. The pretreatment time (60 min) before testing APIPPS was established in our pilot study as the time to peak of maximum anticonvulsant activity of APIPPS (unpublished data).

Electroconvulsions. Electroconvulsions were induced by applying an alternating current (50 Hz; 500 V) *via* ear-clip electrodes from a rodent shocker generator (type 221; Hugo Sachs Elektronik, Freiburg, Germany). The stimulus duration was 0.2 s. Tonic hind limb extension was used as the endpoint. This apparatus was used to induce seizures in two methodologically different experimental approaches: maximal electroshock seizure threshold (MEST) test and maximal electroshock seizure (MES) test [5,6].

Maximal electroshock seizure threshold test. The MEST test was first used to assess the anticonvulsant effects of APIPPS administered alone. In this test, at least 4 groups of control mice, each consisting of 8 animals, were challenged with currents of varying intensities ranging between 5 and 8 mA so that 10–30%, 30–50%, 50–70% and 70–90% of animals exhibited the endpoint. After establishing the current intensity-effect curve (i.e., current intensity in mA *vs.* percentage of mice convulsing) for each dose of APIPPS tested, the electroconvulsive threshold was calculated according to the log-probit method of Litchfield and Wilcoxon [4]. The electroconvulsive threshold was expressed as the median current strength value (CS_{50} in mA) predicted to produce tonic hind limb extension in 50% of the animals tested. This experimental procedure was performed for various increasing doses of APIPPS (25, 50, 100 and 150 mg/kg), until the threshold for electroconvulsions of APIPPS-injected animals was statistically different from that of the control animals.

Only doses of APIPPS that did not significantly affect the seizure threshold in the MEST test were selected for testing in combination with four classical AEDs in the MES test (see below). This approach allowed us to rule out any contribution of the intrinsic anticonvulsant efficacy of APIPPS in the effects observed in combination with the AEDs in the MES test.

Maximal electroshock seizure test. In the MES test, mice were challenged with a current of the fixed intensity (25 mA) that was 4-5-fold higher than the CS_{50} value in vehicle-treated control mice [5,6]. These parameters of stimulation (maximal electroshock) typically result in all mice responding with tonic hind limb extension immediately after stimulation. The AEDs administered alone and their combination with APIPPS were tested for their ability to increase the number of animals not responding with tonus (i.e., protected from tonic hind limb extension) after stimulation. Again, at least 4 groups of mice, each consisting of 8 animals and treated with a different dose of the AED alone or in combination with APIPPS, were challenged with a current of 25 mA to yield 10 – 30%, 30 – 50%, 50 – 70% and 70 – 90% of animals protected from tonic seizures. After constructing a dose-effect curve (i.e., dose in mg/kg *vs*. percentage of mice protected), the protective median effective dose (ED_{50}) value of the AED tested was calculated according to a log-probit method [4]. Each ED_{50} value represented a dose of the AED (in mg/kg) predicted to protect 50% of mice tested against MES-induced extension of the hind limbs. APIPPS was tested for its ability to affect the anticonvulsive potency of antiepileptic drugs. As mentioned earlier, APIPPS was administered in doses that *per se* had no effect on seizure threshold in the MEST test. In this experimental protocol, an increase in the anticonvulsant potency of the AED tested in combination with APIPPS would be reflected by a lower ED_{50} value of the test AED (i.e., lower dose of test drug was necessary to protect 50% of mice challenged). In the present study, CBZ and PHT were administered at doses ranging between 4–12 mg/kg, PB at doses ranging between 10–30 mg/kg and VPA at doses ranging between 125–275 mg/kg.

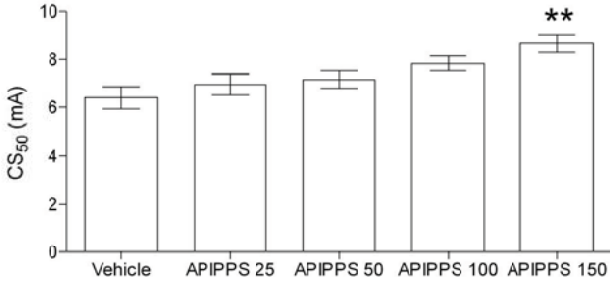
Measurement of total brain antiepileptic drug concentrations. Pharmacokinetic evaluation of total brain AED concentrations was performed only for those combinations of APIPPS with AEDs for which the anticonvulsant effect in the MES test was significantly greater than that for control (an AED + vehicle-treated) animals. Thus, the measurements of total brain concentrations of PB and VPA were undertaken at the doses that corresponded to their ED_{50} values from the MES test. Specifically, mice pretreated with a given AED alone or in combination with APIPPS were decapitated at times reflecting the peak of maximum anticonvulsant effects for the drugs in the MES test. The whole brains of mice were removed from skulls, weighed, harvested and homogenized using Abbott buffer (1:2 weight/volume; Abbott Laboratories, North Chicago, IL, USA) in an Ultra-Turrax T8 homogenizer. The homogenates were then centrifuged at 10,000 *g* for 10 min and the supernatant samples of 100 μl were collected and then analyzed for AED content. Total brain concentrations of PB and VPA were measured by a fluorescence polarization immunoassay using an analyzer (Abbott TDx) and manufacturer-supplied reagent kits (Abbott Laboratories, North Chicago, IL, USA). Total brain AED concentrations are expressed in μg/ml of brain supernatants as means ± standard error (S.E.) of at least 8 separate brain preparations.

Statistics. Both CS_{50} and ED_{50} values with their 95% confidence limits were calculated by computer log-probit analysis according to Litchfield and Wilcoxon [4]. Subsequently, the respective 95% confidence limits were transformed to S.E. as described previously [7]. Statistical analysis of data from the MEST test was performed with one-way analysis of variance (ANOVA) followed by the post-hoc Tukey-Kramer test for multiple comparisons among five CS_{50} values. Statistical analysis of data from the MES test was performed with one-way ANOVA followed by the post-hoc Tukey-Kramer test for multiple comparisons among three or four ED_{50} values. Total brain AED concentrations were statistically compared using the unpaired Student's *t*-test. Differences among values were considered statistically significant if $p<0.05$. All statistical tests were performed using commercially available GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

INFLUENCE OF APIPPS ON THE THRESHOLD FOR ELECTROCONVULSIONS. APIPPS administered systemically (*i.p.*, 60 min prior to the test) at a dose of 150 mg/kg significantly elevated the threshold for electroconvulsions in mice from 6.40 mA to 8.68 ($p<0.01$; Figure 1). The experimentally-derived CS_{50} values for animals receiving APIPPS at doses of 25, 50 and 100 mg/kg did not significantly differ from that for control animals subjected to the MEST test (Figure 1).

EFFECTS OF APIPPS ON THE PROTECTIVE ACTION OF CARBAMAZEPINE, PHENOBARBITAL, PHENYTOIN AND VALPROATE IN THE MOUSE MAXIMAL ELECTROSHOCK SEIZURE MODEL. When 100 mg/kg APIPPS was co-administered with PB, it significantly enhanced the anticonvulsant action of the latter drug in the MES test by reducing the ED_{50} value of PB from 23.3 mg/kg to 13.0 mg/kg ($p<0.01$; Figure 2A). In contrast, 50 mg/kg APIPPS had no significant impact on the anticonvulsant action of PB against MES-induced seizures in mice (Figure 2A). Moreover, when APIPPS at

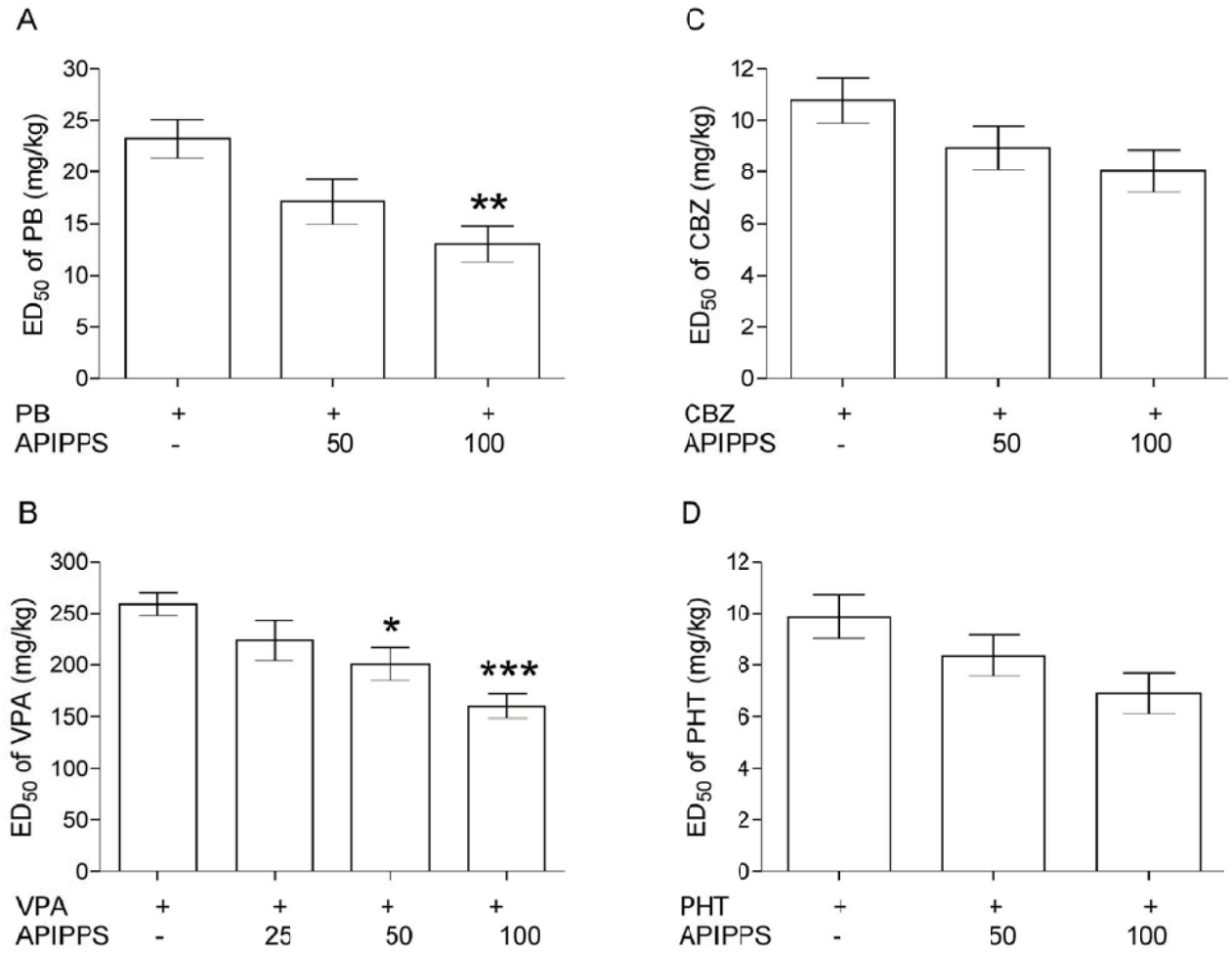


Columns represent median current strengths (CS_{50} values ± S.E. as the error bars) required to produce tonic hindlimb extension in 50% of animals tested in the MEST test. APIPPS was administered i.p. 60 min. before the test. Statistical evaluation of the data was performed with log-probit method and one-way ANOVA followed by the post-hoc Tukey-Kramer test for multiple comparisons. **p<0.01 vs. the control (vehicle-treated) animals.

Fig. 1. Effect of N-(p-acetylphenyl)-p-isopropoxyphenyl-succinimide (APIPPS) on the threshold for electroconvulsions in mice

(p<0.001), respectively (Figure 2B). APIPPS at the dose of 25 mg/kg did not significantly affect the antielectroshock action of VPA in mice (Figure 2B). Similarly, APIPPS at doses of 50 and 100 mg/kg did not significantly alter the anticonvulsant action of CBZ or PHT in the MES test in mice (Figures 2C and 2D).

INFLUENCE OF APIPPS ON TOTAL BRAIN ANTIEPILEPTIC DRUG CONCENTRATIONS. As determined by the fluorescence polarization immunoassay method, 100 mg/kg APIPPS did not significantly affect the total brain concentration of PB co-administered at a dose of 13.0 mg/kg (Figure 3A). Similarly, 100 mg/kg APIPPS did not significantly affect the total brain concentration of VPA co-administered at a dose of 160.1 mg/kg (Figure 3B).



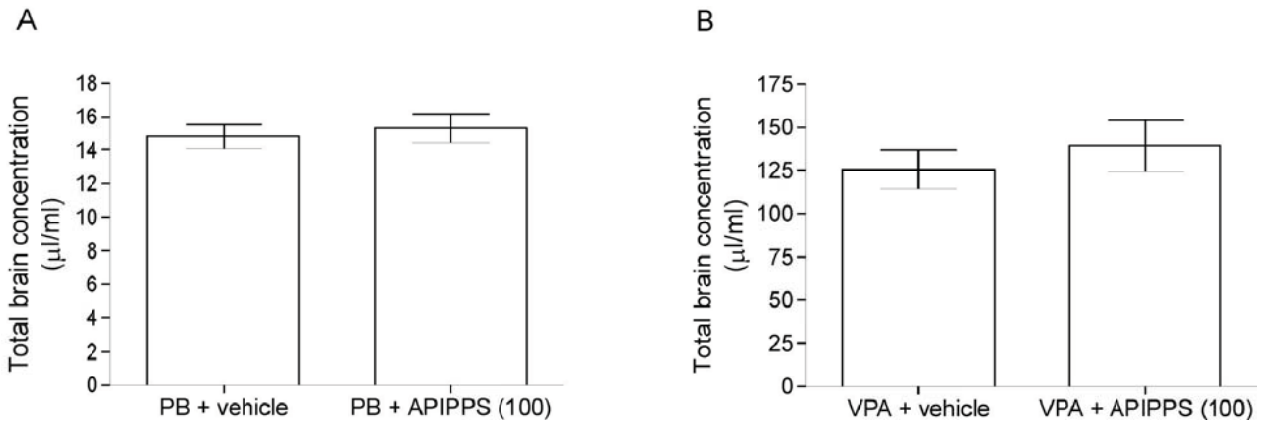
Columns represent median effective doses (ED_{50} in mg/kg ± S.E. as the error bars) of AEDs, protecting 50% of animals tested against MES-induced hindlimb extension. All AEDs were administered i.p.: PHT – 120 min., PB – 60 min., CBZ and VPA – 30 min. prior to the MES test. APIPPS was administered i.p. at 60 min. before the MES test. Statistical analysis of data was performed with one-way ANOVA followed by the post-hoc Tukey-Kramer test for multiple comparisons. CBZ – carbamazepine, PB – phenobarbital, PHT – phenytoin, and VPA – valproate. *p<0.05, **p<0.01 and ***p<0.001 vs. control (AED + vehicle-treated) animals.

Fig. 2A-D. Effects of N-(p-acetylphenyl)-p-isopropoxyphenylsuccinimide (APIPPS) on the protective activity of four classical antiepileptic drugs against maximal electroshock-induced seizures in mice

doses of 50 and 100 mg/kg was co-administered with VPA, it significantly enhanced the anticonvulsant action of the latter drug by reducing the ED_{50} value of VPA from 259.3 mg/kg to 200.5 mg/kg (p<0.05) and 160.1 mg/kg

DISCUSSION

Results presented herein indicate that APIPPS elevated, in a dose-dependent manner, the threshold for electroconvulsions in mice. Moreover, the compound at



Columns represent mean concentrations (in μg/ml ± S.E. as the error bars of 8 determinations) of AEDs in the brain tissue. Statistical evaluation of data was performed with unpaired Student's *t*-test. Brain tissue samples were taken at times scheduled for the MES test and the total brain AED concentrations were quantified using fluorescence polarization immunoassay.

Fig. 3A-B. Brain concentrations of antiepileptic drugs administered singly or in combination with N-(p-acetylphenyl)-p-isopropoxyphenylsuccinimide (APIPPS)

the sub-protective dose of 100 mg/kg (the dose that by itself did not significantly affect the threshold for electroconvulsions) potentiated the anticonvulsant activity of PB and VPA against MES-induced seizures in mice. Moreover, APPIPS at a dose of 50 mg/kg significantly potentiated the anticonvulsant action of VPA, but not that of PB in the mouse MES model. In contrast, APIPPS at the sub-protective doses of 50 and 100 mg/kg had no significant impact on the antielectroshock action of CBZ and PHT in mice, thus indicating neutral interactions between these drugs in the mouse MES model. The combinations of APIPPS with PB and VPA were pharmacodynamic in nature because APIPPS did not significantly alter total brain PB or VPA concentrations in experimental animals. It is noteworthy that in this study, total brain AED concentrations were verified with fluorescence polarization immunoassay technique because, as reported earlier, only total brain concentrations of AEDs provide the exact classification and characterization of interactions between AEDs [1,11].

Comparing the effects produced by APIPPS with those reported earlier for AMIPPS (an N-(anilinomethyl)-substituted IPPS), one can ascertain that both p-isopropoxyphenylsuccinimide derivatives possess identical profiles when co-administered with CBZ, PB, PHT and VPA in the mouse MES model. However, it has been reported that the observed interaction between AMIPPS and VPA was complicated by a significant pharmacokinetic increase in total brain VPA concentration [10], whereas, in the present study, APIPPS had no impact on the total brain VPA concentration and thus, the observed interaction was pharmacodynamic in nature.

Moreover, it has been found in the mouse MES model that IPPS enhanced the anticonvulsant action of PHT and VPA, but not that of CBZ and PB [9]. In contrast, o-CAMIPPS reduced the anticonvulsant action of CBZ, but not that of PB, PHT and VPA in the mouse MES model [8]. In case of m-CAMIPPS and p-CAMIPPS, both succinimide derivatives had no impact on the protective action of four classical AEDs (CBZ, PB, PHT and VPA) in the mouse MES model [8]. In light of the above-mentioned facts, the interaction profile of APIPPS is quite similar to AMIPPS in the mouse MES model. It seems that N-(p-acethylphenyl) and N-(anilinomethyl) groups of p-isopropoxyphenylsuccinimide derivatives are responsible for the enhancement of the anticonvulsant action of PB and VPA in the mouse MES model.

At present, it is unknown why APIPPS potentiated the anticonvulsant action of PB, but not that of CBZ and PHT in the mouse MES model. To elucidate this phenomenon, more advanced molecular, neurochemical and electrophysiological studies are required.

CONCLUSION

Based on the results from this study, one can ascertain that the co-administration of APIPPS with classical AEDs, especially with PB and VPA, might be favorable for epileptic patients with tonic-clonic seizure or partial convulsions with or without secondarily generalization, if the results from this study could be extrapolated into clinical settings. Although this hypothesis needs verification in further neurochemical and electrophysiological studies, APIPPS might be considered as a supplementary compound in further clinical settings.

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